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Note

NOTE

The influence of a cryoprotective medium containing glycerol on the lyophilization of lactic acid bacteria

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The aims of liophilization (freeze-drying) of lactic acid bacteria are to preserve pure cultures or to prepare starters for the dairy industry. In both cases, the choice of the cryoprotectant is very important. In this work, samples of *Bifidobacterium breve* A71 and *Bifidobacterium bifidum* BbTD were freeze-dried in a new cryoprotective medium containing lactose, gelatine and glycerol (medium B). The reference medium contained saccharose, gelatine and skim milk (medium A). Before liophilization, the eutectic points of both media were determined, because the products must be cooled to a temperature below its freezing point. The success of the cryoprotectants was estimated in terms of the number of surviving organisms after lyophilization. *Bifidobacterium breve* A71 and *Bifidobacterium bifidum* BbTD freeze-dried in media A and B showed high survival rates. *Bifidobacterium breve* A71 showed a greater percentage survival in combination with medium B than with medium A. These results could be utilized in the manufacture of *Bifidobacterium breve* A71 as a starter in the diary industry because it is a human isolate which, except for acidification, has probiotic activity.

Keywords: lyophilization, lactic acid bacteria, *Bifidobacterium bifidum*, cryoprotectants.

INTRODUCTION

The most satisfactory method for the long term preservation of cultures is lyophilization or freeze-drying under vacuum. This is a stabilizing process in which a solution of a substance is first frozen and then the quantity of the solvent (generally water) is reduced, first by sublimation (referred to as the primary drying process) and then by desorption (secondary drying process) to a value that will no longer support biological activity or chemical reactions.[1] The result of lyopohilization is greatly influenced by cryoprotectants – one or more substances which protect cells membranes against the effects of exposure to low temperature. A large number of different cryoprotectivants have been used for preparing bacterial suspensions before drying: saccharose, lactose, trehalose, glycerol, sodium glutamate, adonitol, peptone, dextran...[2–6] These compo-

nents generally improve the resistance of freezing. Brigs (1955) suggested the noe of a combination of skim milk and 3 % lactose while Lagoda, Bannikova (1974, 1975) suggested 10 % saccharose, 5 % gelatine and 2 % sodium glutamate as cryoprotectants for lactic acid bacteria.[2]

Desmons *et al*.[3] showed that:

– a combination of 10 % skim milk, 5 % glycerol and 0.1 % $CaCO_3$ resulted in 85.9 percentage survival,

– a combination of 10 % skim milk, 5 % saccharose resulted in 67.4 percentage survival.

In this work a combination of 5 % lactose, 1.5 % gelatine and 1 % glycerol was investigated. This is a new combination of cryoprotectants (medium B) in the freeze-drying of lactic acid bacteria, which is based on literature data on particular components.[2,3,4,9] The obtained data were analyzed in terms of the number of surviving organisms and compared with data obtained with a commercial medium – A consisting of saccharose, gelatine and skim milk.[5] Both media contain sugars which act as dehydrating agents reducing the amount of intracellular water.[4] Milk and gelatine are protective coloids.[4] Glycerol, which modifies the rate of crystal growth and membrane permeability,[4,8] was included in medium B.

EXPERIMENTAL

Bacterial strains and their maintenance

Two human isolates of the *Bifidobacterium* genus were used. *Bifidobacterim bifidum* BbTD - byotype a, was provided by the Institute of Immunology and Virology "Torlak", Belgrade. *Bifidobacterium breve* A71 was provided by the Faculty of Technology and Metallurgy, Belgrade.

These strains agreed with the published results of Gram stain, catalase reaction, cell morphology and fermentation of carbohydrates (Kandler, Weiss, Scardovi, 1986).[12]

These strains were cultivated under microaerophilic conditions (Pro Gas – Torlak) in MRS broth – Difco supplemented with lactose – Difco (2 %), L-cystein – BDH(0.5 %), and $CaCl_2$ – Zorka (0.37 %).[9,10,11] The supplemented MRS broth plus 1.5 % agar were used for enumerating the cultures. Also, the growth rates of the cultures were determined in the supplemented broth.

Cryoprotective media

Lactic acid bacteria *Bifidobacterium breve* A71 and *Bifidobacterium bifidum* BbTD were freeze-dried in the two cryoprotective media. Medium A contained 8 % saccharose, 1.5 % gelatine and 10 % skim milk and medium B contained 5 % lactose, 1.5 % gelatine and 1 % glycerol. Medium A was a commercial cryoprotectant used in the freeze-drying of *Bifidobacterium bifidum* BbTD.

The eutectic temperature was determined for both media. Media were placed in a cell which was then plunged into a Thermos flask containing alcohol refrigerated with dry ice to – 60 ºC.

The eutectic temperature was measured by progressively warming the media and simultaneously measuring continuously both the resistance and temperature. The resistance was measured by connecting an electrode with a microampermeter (class 1.5, 0–100 μA, internal resistance 1800 Ω "Metrix" 9). The temperature was measured using a platinum thermometer, connected with a galvanometer (Pt 100 in one arm of a Wheatstone bridge).

Preparation of Cultures for lyophilization

The optical densities of both cultures *vs.* time were measured to obtain growth curves. Special attention was paid to the beginning of the early stationary phase. Many data have confirmed that prior lyophilization the cultures must be harvested in the early stage of the stationary phase.[1,2,6,8]

Bifidobacterium breve A71 and *Bifidobacterium bifidum* BbTD were incubated until the stationary phase (20 h and 22 h in MRS supplemented broth, microaerophilic, at 37 ºC) were reached. The growth of the cells was followed by measuring the pH and optical density of the culture at 580 ηm (pH-meter Iskra MA 5705 and colorimeter Iskra MA 9507). The growth was stopped at about 10^9 CFU/ml.[5,6]

Both cultures in their stationary phase (300 ml) were centrifuged at 3000 rpm. The supernatant was discarded and the concentrated cells were suspended in 300 ml of either media A or B. The obtained mixture was homogenized and the viable cells (CFU/ml) were enumerated in soft MRS agar by the agar plate count method. The plates were incubated under microaerophilic conditions at 37 ºC. The colonies were enumerated and recorded as colony forming units per mililiter of product. The product was placed in glass vials (2.0 ml). The products (bifidobacteria with media A or B) were lyofilized separately, at least three times.

Lyophilization

The first phase of lyophilization was freezing the products in alcohol. The product must be cooled, according to the determination of the eutecticum, to a temperature below the eutectic point. The equipment for freeze-drying was a Lyophilizer USIFROID, SMRG; 1959.

The second phase of lyophilization was primary drying and the third phase was secondary drying. During these phases, the temperature of the products and the temperature of the condenser were measured.

After drying, the vials were sealed under vacuum and stored in a refrigerator. The cultures obtained after lyophilization were diluted. The survival rate of the microorganisms were enumerated in soft MRS agar by the agar plate acount method. Enumeration was carried out using the agar plate count method, as for the samples before lyophilization. This was the method used for quantifying the survival rates of the cultures during lyophilization. The results are arithmetic means of three measurements.

RESULTS AND DISCUSSION

In this paper, a new combination of cryoprotectants for lyophilization of *Bifidobacteria* was introduced. The first step was the examination of the eutectic point of media. The eutecticum of the media were determined by the method of tangents on the curve of the relationship between temperature and resistance (Figs. 1 and 2). The eutecticum for medium A is –22.7 ºC and for medium B is –28.1 ºC, which means that the product must be cooled to about –30 ºC to –35 ºC.

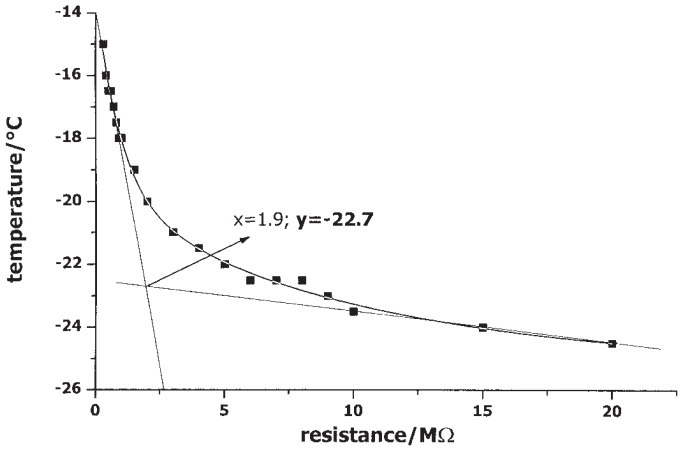

Fig. 1. Determination of the eutecticum of medium A on the curve of the relationship between temperature and resistance.

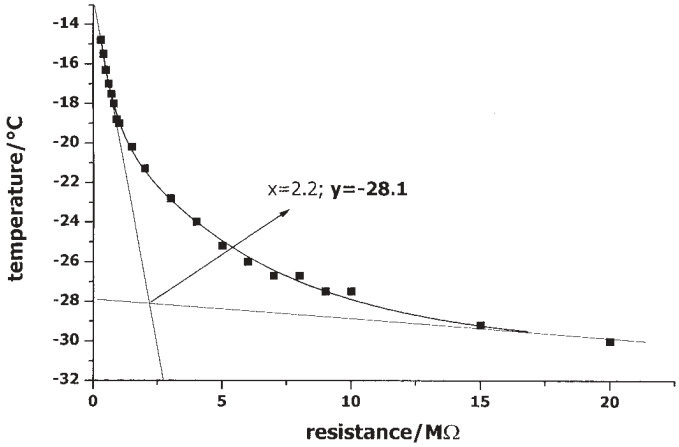

Fig. 2. Determination of the eutecticum of medium B on the curve of the relationship between temperature and resistance.

The cell concentration before lyophilization was about 10^9 CFU/ml. The number of viable cells before and after freeze-drying is summarized in Table I. The survival rate of the cultures after freeze-drying was estimated in terms of the number of surviving microoganisms.

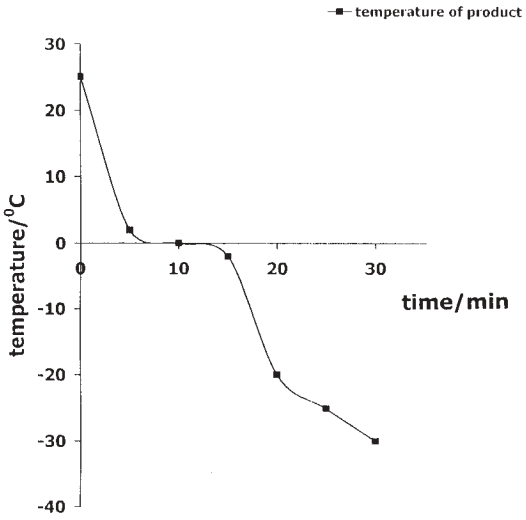

Fig. 3. The curve of the freezing of products in a cryoprotective media A and B.

The first phase of lyophilization – freezing the products – is shown in Fig. 3. The curves of freezing of both cultures (products) have the same shape. Near 0 °C, a plateau occurs on the curve. During this period pure water freezes, and the cryoprotectants are concentrated in the remaining solution.

The primary and secondary drying in the cryoprotective medium A is shown in Fig. 4 and in medium B in Fig. 5. Primary drying is until the product reaches 0 °C. This phase was shorter for medium A (7.1 h) than for medium B (7.5 h). During the primary drying phase, the condenser removes water vapor by vacuum sublimation. The second-

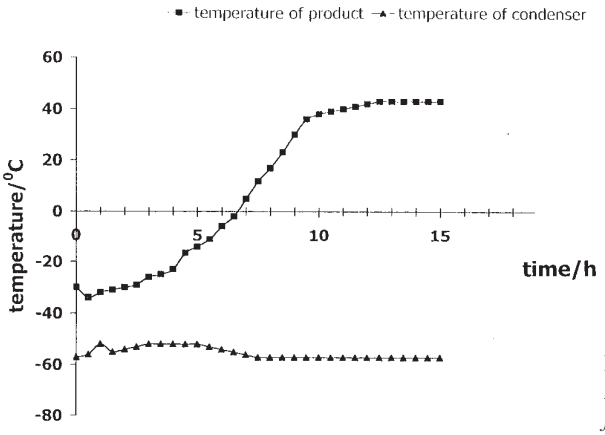

Fig. 4. Time course of freeze-drying of *Bifidobacterium brave* A71 and *Bifidobacterium bifidum* BdTD in a cryoprotective medium A.

ary drying for medium B is longer at a lower temperature (for medium A, the secondary drying temperature is 43 ºC, for medium B is 18 ºC). In this phase, the condenser removes desorbed water vapour from the products.

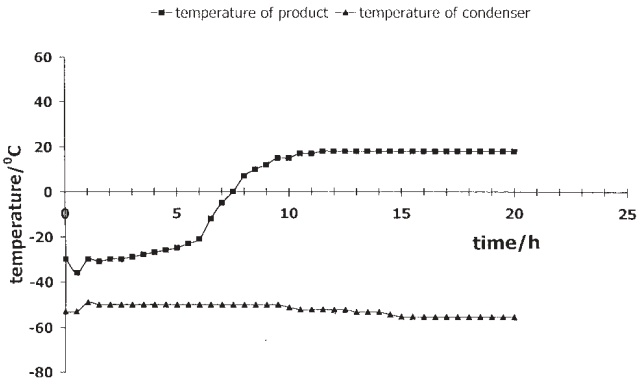

Fig. 5. Time course of freeze-drying of *Bifidobacterium breve* A71 and *Bifidobacterium bifidum* BbTD in a cryoprotective medium B.

The succes of the new combination of cryoprotectants was estimated in terms of the number of surviving organisms after lyophilization. For both strains, the survival rates were slightly better in medium B than in medium A. The obtained results were expected since glycerol has already been recognized as a cryoprotective agent for lactic acid bacteria during freezing.[8] It was observed that the new cryoprotective media gave better results in the freeze-drying of *Bifidobacterium breve* A71 (83.3 %) than in the freeze-drying of *Bifidobacterium bifidum* BbTD (73.9 %) – Table I. Probably this result is due to intrinsic differences in the strains mentioned above.[11]

TABLE I. Survival rate of *Bifidobacteria breve* A71 and *Bifidobacterium bifidum* BbTD during lyophilization in protective media:

A) 8 % saccharose, 10 % skim milk and 1.5 % gelatin
B) 5 % lactose, 1.5 % gelatin and 1 % glycerol

Culture	Media	Cultures before centrif.		Viability before lyophilization	Viability after lyophilization	Survival rate
		pH	O.D.	CFU/ml	CFU/ml	%
A71	A	3.85	1.10	3.6×10^9	2.4×10^9	66.6
	B	3.87	1.17	3.6×10^9	3.0×10^9	83.3
BbTD	A	3.87	1.15	4.4×10^9	3.0×10^9	68.2
	B	3.90	1.20	4.6×10^9	3.4×10^9	73.9

CONCLUSION

Lactic acid bacteria are of great importance in the dairy and pharmaceutical industry. The best method for preserving these cultures is lyophilization with optimization of the cryoprotectants, cells concentration and process conditions (in conformity with the equipment). In this method, the cultures should be harvested in the early stationary phase and then mixed with the cryoprotective media. *Bifidobacterium breve* A71 in the cryoprotective medium with grycerol had a percentage survival of 83.3. The resistance of freezing is improved by adding a cryoprotective agents such as glycerol.

In this experiment pure lyophilized cultures of *Bifidum bacterium breve* A71 for long-term storage were obtained. Also, these results could be utilized in the manufacture of *Bifidobacterium brave* A71 as a starter in the diary industry because it is a human isolate which has acidifiaction and probiotic activity.[7]

И З В О Д

УТИЦАЈ КРИОПРОТЕКТИВНОГ МЕДИЈУМА СА ГЛИЦЕРОЛОМ НА ЛИОФИЛИЗАЦИЈУ БАКТЕРИЈА МЛЕЧНЕ КИСЕЛИНЕ

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Бактерије млечне киселине се лиофилизују (суше у замрзнутом стању) са циљем чувања култура на дуже време или у форми стартера за млечну индустрију. У оба случаја важан је избор криопротектаната. У овом раду праћена је лиофилизација *Bifidobacterium breve* A71 и *Bifidobacterium bifidum* BbTD у новом криопротективном медијуму са лактозом, желатином и глицеролом (медијум B). Референтни медијум садржи сахарозу, желатин и обрано млеко (медијум A). Лиофилизацији претходи одређивање еутектичке температуре јер се фаза замрзавања одвија на нешто нижој температури од еутектичке. Ефикасност криопротектаната испитивана је у смислу преживљавања култура након лиофилизације. *Bifidobacterium breve* A71 и *Bifidobacterium bifidum* BbTD лиофилизовани у медијумима A и B остају у високом проценту вијабилне.

Обе комбинације криопротектаната дају релативно висок проценат преживљавања. *Bifidobacterium breve* A71 у комбинацији са медијумом B има већи проценат преживљавања него у комбинацији са медијумом A. Ови резултати могу постужити у производњи лиофилизованог *Bifidobacterium breve* A71 – стартера у млечној индустрији јер је то хумани изолат са потенцијално добрим пробиотским особинама.

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